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Review

MAP kinases in chondrocyte differentiation

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Abstract

The majority of the vertebrate skeleton develops through the process of endochondral ossification and involves successive steps of chondrogenesis, chondrocyte proliferation, and hypertrophic chondrocyte differentiation. Interruption of this program through gene mutations and hormonal or environmental factors contributes to numerous diseases, including growth disorders and chondrodysplasias. While a large number of growth factors and hormones have been implicated in the regulation of chondrocyte biology, relatively little is known about the intracellular signaling pathways involved. Recent data provide novel insights into the mechanisms governing acquisition of new phenotypes within the chondrogenic program and suggest multiple pivotal roles for members of the mitogen-activated protein kinase family and their downstream targets in cartilage development. These data are summarized and discussed here.

Chondrogenesis and endochondral ossification

The vertebrate skeleton is the end result of the well-orchestrated and intricately controlled multistep differentiation of mesenchymal cells (Cancedda et al., 1995, 2000; Olsen et al., 2000). Three cell populations contribute to the vertebrate skeleton. Neural crest cells give rise to craniofacial bones, while the axial skeleton forms from the sclerotome compartment of the somites. The lateral plate mesoderm yields the skeleton of the limbs. Each of these skeletal elements is prefigured in mesenchymal condensations between 10.5 and 12.5 days postcoitum (dpc) in mice, whereafter they can follow two pathways. The first being differentiation of condensed cells directly into osteoblasts in a process termed intramembraneous ossification. These events result in the majority of the craniofacial bones and the clavicles. In the remaining part of the future skeleton, condensation of mesenchymal progenitor cells is followed by differentiation of these cells into chondrocytes, in a process termed chondrogenesis (Fig. 1). In a second step, cartilage is replaced by bone tissue and bone marrow to give rise to mature bones. The involvement of a cartilage intermediate in this second process leads to the term endochondral ossification.

Chondrogenesis is initiated by the condensation of mesenchymal precursor cells and is accompanied by expression of molecules involved in cell–cell adhesion such as N-cadherin (DeLise et al., 2000) (Fig. 2). This is followed by the expression of chondrocyte-specific genes such as the transcription factor Sox9 and genes encoding cartilage extracellular matrix proteins (e.g., collagen II, aggrecan). Cells within the centers of these cartilage elements differentiate further to become hypertrophic chondrocytes, which are characterized by their large cell volume and expression of specific marker genes such as collagen X (Kielty et al., 1985; Reichenberger et al., 1991). The formation of a growth plate adjacent to the zone of hypertrophy follows. In the growth plate, chondrocytes proliferate mostly in a unidirectional manner along the longitudinal axis of the later bone, resulting in longitudinal bone growth. Under the control of several growth factors, chondrocytes eventually exit the cell cycle and start to differentiate to hypertrophic chondrocytes. Late hypertrophic chondrocytes initiate the expression of many genes shared with osteoblasts (such as matrix metalloproteinase 13 and bone sialoprotein; (Chen et al., 1991; Gack et al., 1995) and mineralize their extracel-

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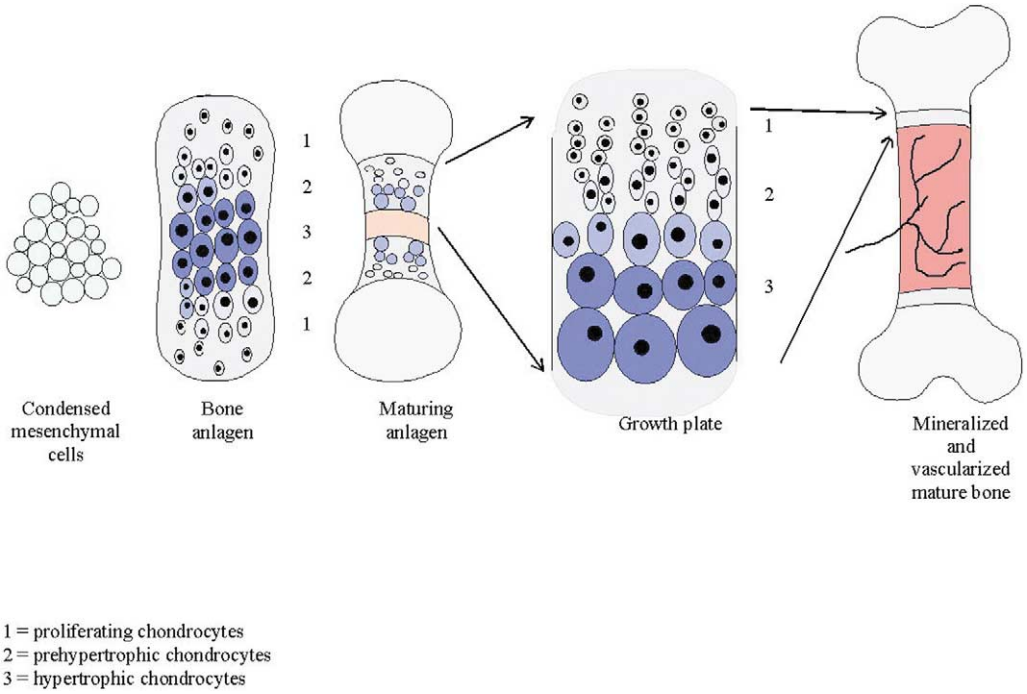

Fig. 1. Endochondral ossification. Endochondral ossification is initiated by the condensation of mesenchymal precursor cells and differentiation of these cells into chondroblasts, giving rise to cartilage anlage of the latter bones. Cells in the centre of the anlagen differentiate further to hypertrophic chondrocytes, and growth plates are formed at both sides of the hypertrophic zone. Mineralisation and vascular invasion of hypertrophic cartilage and apoptosis of hypertrophic chondrocytes follow. Hypertrophic cartilage is then replaced by bone tissue. Growth plates continue to direct longitudinal growth of endochondral bones through proliferation and hypertrophy of chondrocytes.

lular matrix. The fate of the hypertrophic chondrocytes is debated since both apoptosis and further differentiation of the cells into osteoblast-like cells has been described (Adams and Shapiro, 2002). However, it is generally accepted that hypertrophic cartilage is degraded and replaced by bone tissue, completing the process of endochondral ossification. Endochondral ossification therefore consists of distinguishable phases of cell condensation, chondrogenesis, chondrocyte proliferation, hypertrophic chondrocyte differentiation, and replacement of cartilage by bone tissue.

A large number of growth factors and hormones are known to regulate chondrogenesis, chondrocyte proliferation, and hypertrophic differentiation (Fig. 1). These include insulin-like growth factors (IGFs), transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs), parathyroid hormone-related peptide (PTHrP), indian hedgehog, fibroblast growth factors (FGFs), and retinoic acid (Cancedda et al., 1995; DeLise et al., 2000; Wagner and Karsenty, 2001). Disruption of the signaling from many of these factors results in various disease conditions, such as chondrodysplasias, inherited diseases characterized by skeletal deformities and dwarfism (Mundlos and Olsen, 1997a 1997b). For example, activating mutations in the FGF receptor3 gene lead to decreased bone growth in human achondroplasia and hypochondroplasia patients (Bellus et al., 1995; Shiang et al., 1994). Growth plate activity also controls linear growth and final length/height in vertebrates (Frost and Schonau, 2001; Hunziker, 1994; Price et al., 1994). Numerous growth abnormalities (e.g., as a result of endocrine disorders or drug treatments) are associated with disruption of normal growth plate function (Nilsson et al., 1994; Ohlsson et al., 1993; Siebler et al., 2001, 2002; van Leeuwen et al., 2000).

As in many other developmental programs, the timely intracellular relay of extracellular signals is essential for proper bone development. As outlined above, the number of extracellular signals implicated in this process is continuously expanding. In addition, Sox9 and Cbfa1 have been identified as important transcriptional regulators of early and late chondrogenic differentiation, respectively (de Crombrugghe et al., 2001; Karsenty, 2001). In contrast, relatively little is known about the intracellular signaling pathways controlling chondrogenesis and chondrocyte differentiation. Mitogen-activated protein kinases (MAPKs) are among the most widespread and most studied signaling pathways in eukaryotic cells. The recent increase in information regarding their involvement in chondrocyte proliferation and differentiation is the focus of this review.

MAP kinase cascades

The mitogen-activated protein kinases (MAPKs) are responsible for the conversion of a large number of extracellular stimuli into specific cellular responses that range from positive and negative roles in cell proliferation, differenti-

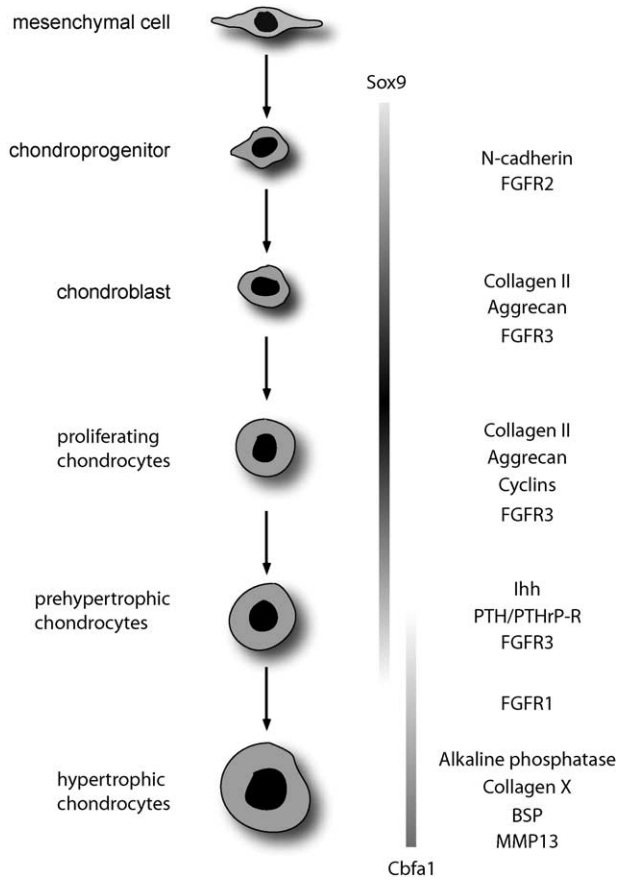

Fig. 2. Schematic representation of the chondrogenic program. Chondrogenesis starts with the condensation of mesenchymal precursor cells, followed by chondrogenic differentiation within the condensations. A subset of chondroblasts then initiates proliferation and subsequently undergoes hypertrophic differentiation. Each stage is characterized by expression of specific marker genes shown on the right. In particular, the expression pattern of two important transcriptional regulators of chondrocyte differentiation, Sox9 and Cbfa1, is indicated.

ation, and apoptosis to regulation of inflammatory and stress responses. The general biochemistry and biology of MAP kinases are the subject of several recent reviews and are therefore only briefly summarized here (Chen et al., 2001; Cobb, 1999; Johnson and Lapadat, 2002; Kyriakis and Avruch, 2001; Pearson et al., 2001; Robinson and Cobb, 1997; Schramek, 2002; Treisman, 1996). MAPK pathways also coordinate regulation of gene transcription and protein synthesis as well as adaptation to changes of the extracellular environment. Multiple MAPK pathways exist in eukaryotic cells (Fig. 3). These pathways are activated by distinct stimuli, including hormones and growth factors that exert their effects through a diverse array of receptor families such as receptor tyrosine kinases, cytokine receptors, G protein-coupled seven-transmembrane receptors, and serine-threonine kinase receptors. It can therefore be appreciated that the MAPK pathways exert a profound effect on cell physiology (for review, see Kyriakis and Avruch, 2001; Pearson et al., 2001).

All of the MAPK pathways are organised into cascades that work in series to result in activation of the MAPK following the concurrent phosphorylation of tyrosine (Tyr) and threonine (Thr) residues within the conserved Thr-X-Tyr motif in the activation loop of the kinase domain. The cascade originates with the phosphorylation of several protein kinase families that are collectively referred to as the MAPK-kinase-kinases (MAP3Ks) or MEK kinases (MEKKs) (Hagemann and Blank, 2001; Kyriakis and Avruch, 2001). These proteins in turn lead to activation of a family of dual specificity kinases referred to as MAPK/extracellular signal-regulated kinase kinases (MEKS or MKKs; MAP2Ks), and it is this family that catalyses the phosphorylation and activation of the MAPKs. Activation of MAP kinases is often accompanied by translocation to the nucleus. MAP kinase substrates include cytoplasmic proteins such as heat shock proteins as well as various transcription factors and downstream kinases.

Three major MAPK families have been identified to date in mammalian cells. They have been categorised according to the amino acid (X) in the dual phosphorylation site (TXY). These families are (1) ERK1 and ERK2, often referred to as p44 and p42 respectively (X = Glu); (2) JNK (JNK1/2/3) or SAPK (X = Pro); and (3) p38 MAPK (p38α/β/γ/δ) (X = Gly). Additional MAP kinases such as ERK5 have been described, but less is known about their function

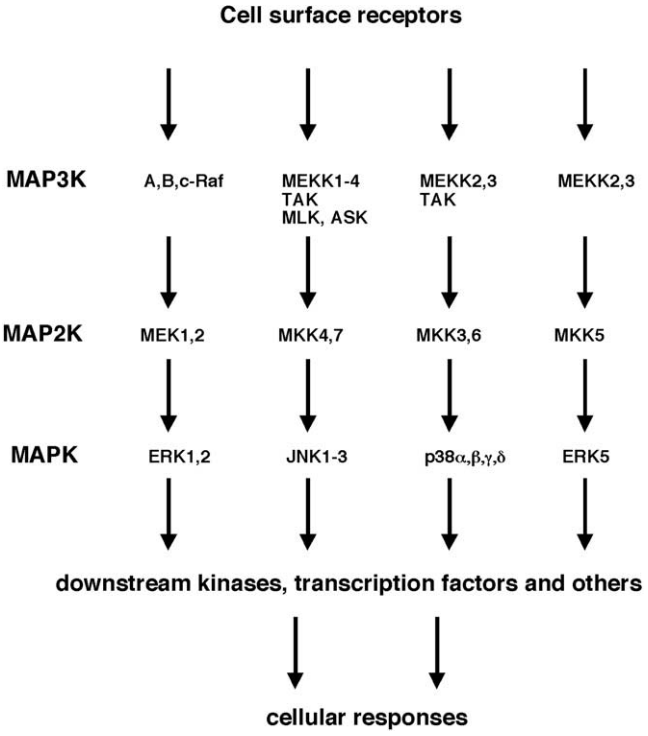

Fig. 3. Organization of mammalian MAP kinase pathways. Following stimulation of receptors, phosphorylation of MAP3K leads to MAP2K phosphorylation and activation that subsequently results in MAPK phosphorylation and activation. Phosphorylation of appropriate substrates and transcription factors causes specific biological responses such as changes in cell proliferation and differentiation or gene expression.

Table 1
MAPK nomenclature

Name	Alternate names	Unigene name	Unigene cluster	Locuslink number	OMIM	Activating stimuli
ERK1	p44-MAPK	MAPK3	Ms. 861	5595	601795	Mitogens
ERK2	p42-MAPK	MAPK1	Ms. 3244T3	5594	176948	Mitogens
JNK1	SAPK-γ, SAPK1c	MAPK8	Ms. 267445	5599	601158	Inflammatory cytokines, stress (e.g. UV, ionizing radiation antibiotics)
JNK2	SAPK-α, SAPK1a	MAPK9	Ms. 246857	5601	602896	Inflammatory cytokines, stress (e.g. UV, ionizing radiation, antibiotics)
JNK3	SAPK-β, SAPK1b, p49F12	MAPK10	Ms. 151051	5602	602897	Inflammatory cytokines, stress (e.g. UV, ionizing radiation antibiotics)
p38α	SAPK2a, CSBP1, MPK2	MAPK14	Ms. 79107	1432	600289	Inflammatory cytokines, stress (osmotic shock, oxidative stress, anisomycin)
p38β	SAPK2b, p38-2	MAPK11	Ms. 57732	5600	602898	Inflammatory cytokines, stress (osmotic shock, oxidative stress, anisomycin)
p38γ	SAPK3, ERK6	MAPK12	Ms. 55039	6300	602399	Inflammatory cytokines, stress (osmotic shock, oxidative stress, anisomycin)
p38δ	SAPK4	MAPK13	Ms. 178695	5603	602899	Inflammatory cytokines, stress (osmotic shock, oxidative stress, anisomycin)
ERK5	SAPK5, BMK1	MAPK7	Ms. 3080	5598	602521	Stress, growth factors

and regulation. The MAPK nomenclature, commonly accepted names and activating stimuli are summarized in Table 1.

Each of the MAPKs are activated by specific MAPKKs: MEK 1/2 for ERK 1/2, MKK3/6 (and possibly MKK4) for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5 (Fig. 3). The complexity and diversity of the MAPK signaling pathways is further enhanced due to the activation of each MAPKK by more than one MAPKKK, thus positioning MAP kinase modules as focal points of signal convergence and crosstalk, MAPKKKs themselves are regulated by multiple mechanisms (Cobb, 1999; Pearson et al., 2001). In particular, small GTPases of the Ras family play important roles in the activation of Raf proteins (the MAPKKKs of the ERK pathway) by growth factor receptors, whereas members of the Rho GTPases family have been implicated in activation of the JNK and p38 proteins. These small GTPases therefore also contribute to signal integration and crosstalk between different MAP kinase modules.

ERK

The first mammalian MAPK pathway to be described was that of the extracellular signal regulated kinases (ERKs). Activation of this pathway has subsequently been observed in a diverse range of cellular activities including cell proliferation. Regulation of the ERK signaling pathway occurs largely through the monomeric GTPase Ras which recruits MAP3Ks of the Raf family to activate MEK1 and MEK2. These MEKs in turn activate the ERKs (for reviews, see Avruch et al., 1994; Marais and Marshall, 1996; Roovers and Assoian, 2000). ERK substrates include the kinases MSK1/2 and MNK1/2 and the transcription factors Elk-1 and SAP1.

Three Raf proteins have been described in mammals with the ubiquitously expressed Raf-1 (c-Raf) having been the most extensively studied. A-Raf and B-Raf expression profiles are more limited. All three Raf enzymes however share Ras as the common upstream activator while MEK serves as the commonly accepted substrate (Morrison and Cutler, 1997; Schaeffer and Weber, 1999). The diverse biological responses activated through this pathway remains puzzling, but may depend on the strength and/or duration of ERK activation (Cook et al., 1999; Marshall, 1995; Woods et al., 1997).

JNK

In contrast to the ERKs, the stress-activated protein kinases (SAPK) or c-Jun NH2-terminal kinases (JNK) (originally termed p54 or p54-MAP2 kinase) are not strongly activated by mitogens, such as epidermal growth factor

(EGF), but are stimulated in response to inflammatory cytokines of the tumour necrosis factor (TNF) family (TNF, interleukin-1, CD-40, CD27 ligand, Fas ligand, receptor activator of NF-κB, RANK ligand etc), by vasoactive peptides (endothelin and angiotensin II), and by a variety of noxious treatments, such as heat shock, ionising radiation, oxidant stress, DNA damaging chemicals, reperfusion injury, mechanical shear stress, and of course, protein synthesis inhibitors (Kyriakis and Avruch, 1996; Kyriakis et al., 1994; Pombo et al., 1994). In addition, JNK activation by TGF-β has been reported (Atfi et al., 1997;) Hocevar et al., 1999; Wang et al., 1997). Upstream activation of JNK occurs via the MAPKKs MKK4 and MKK7. The MAP3Ks for the JNK pathway include MEKKs, mixed lineage kinases (MLKs including MLK1–3 and DLK), Tp12, ASK, and TAK (Davis, 2000; Weston and Davis, 2002).

Ten JNK isoforms, encoded by three different genes, have been identified in mammalian cells. These isoforms differ in expression patterns and interactions with downstream targets (Gupta et al., 1996). The biological roles of JNK include the regulation of stress responses and inflammation, as well as the control of cell proliferation, differentiation and apoptosis (Davis, 2000; Weston and Davis, 2002). Interruption of JNK signaling in genetically engineered mice results in defects mostly in the immune and nervous systems (Pearson et al., 2001).

p38

The third family of mammalian MAPKs is the p38 MAPK family. p38 MAPK was first described by Han et al., (1993, 1994) in murine cells exposed to bacterial lipopolysaccharide (LPS). Currently, four different p38 genes are known, some of which undergo alternative splicing. The various p38 proteins differ in their specificity both for upstream activators and downstream substrates. p38 MAP kinases are activated by a large array of extracellular stimuli, including cytokines, growth factors, stress, and integrins (Adams et al., 2001; Gallo and Johnson, 2002; Nebreda and Porras, 2000; Ono and Han, 2000; Pearson et al., 2001; Shi and Gaestel, 2002). While p38 kinases were originally described as stress- and inflammation-related kinases, recent evidence suggest multiple physiological roles in cell cycle control and in cell proliferation, differentiation and apoptosis (Ambrosino and Nebreda, 2001; Nebreda and Porras, 2000; Pearson et al., 2001). p38 kinases also play crucial roles in the regulation of gene expression through control of transcription (Raingeaud et al., 1996) as well as RNA (Dean et al., 1999; Ridley et al., 1998; Winzen et al., 1999) and protein stability (Bulavin et al., 1999; Casanovas et al., 2000).

p38 MAP kinases are activated by the upstream kinases MKK3 and MKK6. Phosphorylation of p38 by MKK4 is controversial, but analyses of MKK4-deficient mice suggest that MKK4 involvement in p38 regulation might be cell type- and stimulus-specific (Ganiatsas et al., 1998). Numerous p38 substrates have been reported. These encompass various kinases such as MAPKAP K2/3, p38-regulated/activated protein kinase (PRAK), ribosomal S6 kinase-B (RSK-B), mitogen and stress-activated kinase 1 (MSK1)/RLPK, and MAP kinase-interacting kinase 1 and 2 (MNK1/2), together with transcription factors, such as myocyte enhancer factor 2 (MEF2), activation transcription factor-2 (ATF-2), C/EBP homologous protein (CHOP), SAP-1A, and Elk-1, as well as cytosolic proteins such as stathmin (Fig. 3).

p38 MAPKs are the targets of a class of pyridinylimidazole anti-inflammatory drugs, the cytokine-suppressive anti-inflammatory drugs (CSAIDS). Of these, SB203580 has been the most extensively characterised (Cuenda et al., 1995; English and Cobb, 2002) and inhibits p38 through competition with ATP (Young et al., 1997). Only p38α and p38β are inhibited by CSAIDS, while p38γ and p38δ remain completely unaffected (Goedert et al., 1997; Kumar et al., 1997).

MAP kinases in chondrogenesis

Chondrogenesis is initiated by the condensation of mesenchymal precursor cells, which is followed by cell differentiation to chondroblasts and the appearance of cartilage nodules (Cancedda et al., 2000; DeLise et al., 2000). Important signaling pathways controlling these stages during chondrogenesis include the protein kinase C (PKC) and MAPK pathways. Members within this latter pathway, however, demonstrate various degrees of participation in chondrogenesis. For example, JNK phosphorylation is not affected during chondrogenesis, suggesting that JNKs play only minor roles in this process (Nakamura et al., 1999). In contrast, p38s and ERKs occupy central positions in this process. During chondrogenesis of chick mesenchymal cells, p38 phosphorylation is increased and ERK phosphorylation is decreased (Oh et al., 2000). In agreement with this, inhibition of p38 activity blocks chondrogenesis, whereas inhibition of MEK/ERK activity enhances chondrogenesis. Both MAP kinases do not appear to be involved in the initial condensation step, but affect cartilage nodule formation (Oh et al., 2000). Furthermore, inhibition of chondrogenesis by epidermal growth factor (EGF) has been shown to involve upregulation of ERK phosphorylation and inhibition of p38 phosphorylation (Yoon et al., 2000). Similarly, inhibition of chondrogenesis by rapamycin is accompanied by reduced activity of p38 and PKC; however, ERK activity was not affected in these experiments (Oh et al., 2001). Finally, suppression of chondrogenesis by retinoic acid correlates with decreased p38 activity (Weston et al., 2002). These data thus support opposing roles of p38 and ERK in chondrogenesis, with p38 being necessary for chondrogenesis, whereas ERK signaling represses chondrogenesis. However, a recent report demonstrated the requirement for MEK/ERK signaling for the induction of the chondro-

genic master gene Sox9 by FGF signaling (Murakami et al., 2000). Furthermore, stimulation of chondrogenesis of the mouse chondrogenic cell line ATDC5 by growth and differentiation factor 5 (GDF-5), a TGF-β family member, involves both rapid and transient phosphorylation of ERK1 and ERK2, and a slow and sustained phosphorylation of p38. JNK phosphorylation remains unchanged in response to GDF-5 (Nakamura et al., 1999).

In summary, these experiments suggest an obligatory role for p38 in chondrogenesis. p38α and β appear to be of particular importance in this context, since the described experiments relied on the pharmacological inhibitors SB203580 and SB202190, which is specific for these two p38 forms. The role of ERK appears to be more complex and may depend on the intensity or duration of upstream activating signals or localization within the cell.

MAP kinases in chondrocyte proliferation and hypertrophy

In contrast to the roles of ERK and p38 in chondrogenesis, their functions in later stages of chondrocyte maturation are less defined. However, several recent publications have begun to address the roles of p38 and ERK in chondrocyte gene expression, proliferation and hypertrophic differentiation. CTGF/Hcs24 (connective tissue growth factor/hypertrophic chondrocyte specific gene product 24) promotes proliferation and differentiation of chondrocytes in culture. Yosimichi et al. (2001) recently demonstrated that CTGF/Hcs24-induced proliferation of chondrocytes is mediated through the ERK signaling pathway, while CTGF/Hcs24-induced differentiation of chondrocytes is mediated through p38 MAPK. Both ERK and p38 have also been implicated in TGF-β signal transduction in differentiating chondrocytes. TGF-β1 induces a selective, dose-and time-dependent increase in sodium-dependent transport of inorganic phosphate in chondrogenic ATDC5 cells (Palmer et al., 2000). In this cell line, TGF-β1 induces Smad signaling pathways as well as ERK and p38 phosphorylation while JNK phosphorylation remains unaffected. Differences in the activation kinetics of these kinases are evident with ERK activation being rapid and transient, as opposed to p38 that displays a slower and more prolonged activation. Smad2 and ERK activation display similar kinetics but, in contrast to ERK activation (which is only transient), Smad2 phosphorylation is maintained for several hours, similar to p38 MAPK activation.

TGF-β also rapidly induces expression of the proteoglycan aggrecan in ATDC5 cells via the Smad signaling pathway (Watanabe et al., 2001). At early stages of ATDC5 differentiation, TGF-β induced activation of aggrecan gene expression requires Smad signaling as well as activity of ERK and p38 MAP kinases. However, it is interesting to note that following differentiation, aggrecan expression no longer requires TGF-β activated Smads, but is still dependent on both ERK and p38 signaling (Watanabe et al., 2001).

FGFs play multiple roles in the control of chondrocyte proliferation and differentiation (Naski and Ornitz, 1998). ERK phosphorylation is induced by FGF9 and FGF18 in chondrocytes (Rozenblatt-Rosen et al., 2002; Shimoaka et al., 2002). ERK phosphorylation in response to FGF9 is accelerated by thanatophoric dysplasia mutants of the FGF receptor 3; however, although these mutations act in an activated manner, no ligand-independent activation of ERK by FGF receptor 3 was observed (Legeai-Mallet et al., 1998). FGF18 also induces a transient activation of p38, and both ERK and p38 are required for the mitogenic actions of FGF18 on chondrocytes in vitro (Shimoaka et al., 2002). However, it should be noted that the concentrations of the p38 inhibitor SB203580 required for these activities are 10 μM and higher. At these concentrations the inhibitors may affect other pathways as well—an important consideration when using these reagents (Lali et al., 2000). Similar considerations apply to many of the experiments addressing p38 function described here.

The c-Raf kinase, which is a MAP3K for ERK1/2, has been shown to be upregulated during chondrocyte differentiation in vivo (Kaneko et al., 1994). Recent evidence from our laboratory also demonstrated that c-Raf, MEK1/2, and ERK1/2 are required for the normal expression of the collagen X and $p21^{WAF1/CIP1}$ genes, which are markers for hypertrophic chondrocytes (Beier et al., 1999b,c). These data suggest a role of the c-Raf/MEK1/2-ERK1/2 pathway in hypertrophic differentiation. In agreement with that, generation of a hypomorphic allele of c-Raf in mice, where c-Raf activity is reduced to approximately 10% of wild type activity, causes a severe delay in growth and endochondral ossification (Wojnowski et al., 1998). However, it remains to be shown whether this effect is completely due to a general developmental delay or to specific roles of c-Raf in chondrocytes. p38 has been implicated in the control of collagen X transcription as well (Beier and LuValle, 1999). Zhen et al. (2000) have shown that suppression of p38 activity is involved in the effects of parathyroid hormone (PTH) on chicken growth plate chondrocytes. In these experiments, inhibition of p38 signaling resulted in an accumulation of cartilage matrix protein expression, a marker of prehypertrophic chondrocytes, but suppresses expression of collagen X, a marker of hypertrophic chondrocytes. These data suggest that p38 signaling is required for the transition from the prehypertrophic to the fully hypertrophic chondrocyte phenotype.

A recent report suggests that p38, in contrast to ERK and JNK, is activated by retinoic acid in chondrocytes and is necessary for the induction of collagenase 3, Cbfa1, and osteocalcin expression by retinoic acid, which might represent transdifferentiation of chondrocytes to osteoblast-like cells (Jimenez et al., 2001). In contrast, inhibition of the ERK pathway by the MEK1/2 inhibitor PD98059 enhances

the induction of these genes by retinoic acid. These results suggest that p38 might play a supportive and ERK a repressive role in chondrocyte-to-osteoblast transdifferentiation.

Upstream activators of MAP kinases in chondrocytes

Only limited data are available on the extracellular regulators of MAP kinase activity in chondrocytes. As outlined above, TGF-β family members induce p38 and ERK phosphorylation with different kinetics (Nakamura et al., 1999; Palmer et al., 2000; Watanabe et al., 2001). In contrast, p38 phosphorylation appears to be inhibited by PTH (Zhen et al., 2000). Retinoic acid has been shown to lead to decreased p38 activity early in chondrogenesis (Weston et al., 2002), but activate p38 signaling at late stages (Jimenez et al., 2001). Receptor tyrosine kinases are crucial regulators of ERK activity (and potentially other MAP kinases). EGF stimulates ERK phosphorylation and inhibits p38 phosphorylation in chondroprogenitor cells (Yoon et al., 2000). Several other growth factors that activate tyrosine kinase receptors play important roles in chondrocyte physiology, such as insulin-like growth factors (IGFs) and FGFs. As discussed above, FGF signaling has been shown to stimulate ERK and p38 phosphorylation and activity in chondrogenic cells (Legeai-Mallet et al., 1998; Rozenblatt-Rosen et al., 2002; Shimoaka et al., 2002). FGF receptor expression is dynamically regulated during chondrocyte differentiation (Ornitz and Marie, 2002), suggesting that differential activation of MAP kinases (and other signaling pathways) by the different receptors could be responsible for their different biological roles in skeletal development. A recent report also demonstrates that p38 is activated by overexpression of $\alpha 6\beta 1$ integrins in chicken chondrocytes, implicating a role for extracellular matrix signaling in the control of p38 activity in chondrocytes, and that p38 signaling is necessary for collagen II expression in these cells (Segat et al., 2002). In summary, p38 and ERK MAP kinases are targeted by many extracellular signals with crucial functions in cartilage development and therefore likely play important roles in these processes as well.

MAP kinase signaling is also likely involved in the transduction of mechanical signals in cartilage development. Proliferation and differentiation of chondrocytes (Elder et al., 2001; Kantomaa et al., 1994; Wu et al., 2001; Wu and Chen, 2000), as well as endochondral bone growth (Frost and Schonau, 2001), are regulated by mechanical forces. Shear stress and loading have been shown to regulate MAP kinase signaling in articular chondrocytes, potentially through integrin receptors (Hung et al., 2000; Jin et al., 2000; Loeser, 2002; Mobasheri et al., 2002). While no studies have addressed whether similar pathways are active in differentiating chondrocytes, it appears likely that mechanical factors contribute to the regulation of MAP kinase activity in the developing skeleton.

Potential MAP kinase targets in chondrocytes

The substrates of MAP kinases in chondrocytes remain unknown. The p38 and JNK substrate ATF-2 is required for normal growth plate morphogenesis and skeletal development (Reimold et al., 1996). However, ATF-2 appears to be most important for chondrocyte proliferation, in part through the regulation of the cell cycle genes cyclin D1 and cyclin A (Beier et al., 1999a, 2000, 2001). In contrast to the role of p38 in chondrogenesis and hypertrophic differentiation, it is not clear whether p38 (or JNK) is necessary for chondrocyte proliferation, or whether ATF-2 activity is regulated through other mechanisms in this phase of the chondrocyte life cycle. Similarly, AP-1 factors have been shown to regulate chondrocyte proliferation and differentiation (Ionescu et al., 2001b; Kameda et al., 1997; Wang et al., 1992), but a possible involvement of MAP kinases as upstream regulators of AP-1 activity in chondrocytes has not been investigated. A potential binding site for the p38 target MEF2 has been described in the collagen X promoter (Harada et al., 1997). Studies in our lab have identified multiple potential MEF2 binding sites in human and mouse collagen X genes and demonstrated that MEF2 activity is required for full activity of the human collagen X promoter (L-A.S., A. Wall, and F.B., unpublished observations). The roles of the numerous other transcription factors and downstream kinases targeted by MAP kinases in chondrocytes still have to be elucidated. Another target of ERK proteins, the ribosomal S6 kinase 2 (RSK2), is mutated in Coffin-Lowry syndrome (Trivier et al., 1996), a genetic disease characterized by mental retardation, reduced growth, and skeletal and craniofacial malformations (Hanauer and Young, 2002). These findings suggest a role for RSK2 (and its upstream regulators) in human cartilage development, which is in agreement with the roles of the RSK2 targets c-Fos (Ionescu et al., 2001b; Thomas et al., 2000; Wang et al., 1992) and CREB (Beier et al., 1999a, 2001; Ionescu et al., 2001a; Long et al., 2001) in chondrocytes.

Open questions

Current evidence suggests important roles of the ERK and p38 MAP kinases in various phases of endochondral ossification. While not all published data necessarily agree on these roles (in particular for ERK), we have to keep in mind that the exact biological functions of these kinases depend on a large number of parameters, including the nature, intensity, and duration of upstream signals, the cellular localization of the signaling proteins, and crosstalk with other signaling pathways. For example, it has been shown that different levels of activation of c-Raf cause different cellular responses (Sewing et al., 1997; Woods et al., 1997), and that the duration of ERK activation determines the behaviour of PC12 (Marshall, 1995). It is there-

fore plausible that ERK stimulates chondrocyte differentiation under certain conditions and inhibits it under other circumstances, and that ERK proteins (or other signaling molecules) have different roles in different phases of chondrocyte differentiation. The exact roles of the MAP kinases in endochondral ossification therefore have to be studied in more detail, including increased use of in vivo models. Most of the functional data reviewed were obtained through the use of pharmacological inhibitors. While still of great value, these approaches will have to be complemented by genetic strategies, such as targeted gene inactivation, expression of dominant-negative kinase mutants, or siRNA technology. Genetic models, with the exception of the mentioned c-Raf hypomorphic mice (Wojnowski et al., 1998), did not yet yield much insight into the functions of MAP kinase modules in skeletal development, mostly because disruption of genes in MAP pathways often are either early lethal (thereby preventing analyses of skeletal development) or have only very subtle defects, presumably due to genetic redundancy. Generation of double "knockout" mice or cartilage-specific gene disruption will be required for a more complete analyses of MAP kinase function in cartilage development. The use of additional model systems (such as Zebrafish or Xenopus) and novel genomic technologies (e.g., microarrays, use of Fugu rubripes genomic sequences for the identification of regulatory DNA elements, etc.) also promise to provide valuable contributions to a better understanding of MAP kinase function in endochondral ossification.

Furthermore, the identity and function of important upstream regulators, downstream mediators, and target genes of MAP kinases in chondrocytes will have to be investigated. For example, while p38 proteins appear to play multiple roles in chondrocyte differentiation, it is not known which of the multiple upstream kinases (in particular MAP3Ks) control p38 activity in cartilage. Similarly, only very few of the potential substrates of MAP kinase signaling have been studied in cartilage development. It will be of interest whether any of the MAP kinases regulate the activity of Sox9 or Cbfa1 (two very important transcription factors in chondrocyte differentiation) in chondrocytes. Finally, analyses of the roles of other MAP kinases (such as ERK5) in chondrocytes and crosstalk among MAP kinases as well as with other signaling systems will be required for a deeper understanding of skeletal development and associated diseases.

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